REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	Oct 18, 1995	Final 10 Jul 92 - 28 Feb 95

4. TITLE AND SUBTITLE
Identification of Biomarkers in Bacteria by Pyrolysis-Tandem Mass Spectrometry

5. FUNDING NUMBERS
DAAL03-92-G-0285

6. AUTHOR(S)
Kent J Voorhees

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Colorado School of Mines
Golden CO 80401

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
ARO 30533.1-LS

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

A new turbomolecular pump and vacuum manifold were added to a chemical and biological mass spectrometer (CBMS). Detection limit measurements on pyridine, toluene, and methyl salicylate are reported for both the unmodified and modified versions of the CBMS. Comparison of pyrolysis-mass spectra of egg albumin showed that the modified instrument spectrum did not match those from unmodified CBMS instruments. The changes in instrument operating parameters and hardware of the modified instrument are described. Four oral presentations have been made on data generated from this contract.

14. SUBJECT TERMS
Pyrolysis-mass spectrometry bacteria, biomarkers, ion trap mass spectrometers

15. NUMBER OF PAGES
21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

Table of Contents

I. INTRODUCTION

The chemical and biological mass spectrometer (CBMS) project was initiated by the U.S. Army in 1985 to build a field portable instrument that would serve to detect both chemical and biological agents on a battlefield. Teledyne CME with Bruker Franzen was awarded the contract to design and build the instrument. Pyrolysis with an ion trap mass spectrometer as the detector, was chosen as the technique to be used for the biological agents. Since pyrolysis procedures had not commonly employed ion trap mass spectrometric detection, much of the definition of the biodetection protocol has been an on-going parallel effort with the instrument development. The following report discusses the efforts at the Colorado School of Mines to add information on pyrolysis-ion trap mass spectrometry.

The technical objective of the original proposal was to define the ultimate utility of the Bruker Franzen ion trap (CBMS) for pyrolysis as a biodetection system. The following three tasks were proposed to accomplish the stated goal:

Task 1 Modification and Testing of the an Existing CBMS Ion Trap. This phase was outlined to transform a field CBMS into an analytical mass spectrometer. Several modifications including a high efficiency vacuum system were proposed.

Task 2 Pyrolysis with the Trap. Once the field CBMS was modified, this phase was centered around comparing pyrolysis results from the modified CBMS to the results from the field model.

Task 3 Long Term Reproducibility. The goal of this phase was to define the possible deterioration of sensitivity, resolution, and reproducibility of the CBMS as a function of operational contamination.

The research conducted during the grant period changed direction several times during the study due to problems that arose from CBMS field studies conducted by ERDEC. Although different compounds and biomaterials were eventually studied, the final results can be grouped into the three original tasks. The successes and failures associated with each task will be individually discussed.

II. RESULTS

A. Task 1 Modification and Testing of the an Existing CBMS Ion Trap.

1. Unmodified CBMS

A schematic of the chemical and biological mass spectrometer (CBMS) is shown in Figure 1. This instrument is composed of a pyrolyzer, a transfer line, a valving system which contains a silicon membrane, and an ion trap mass spectrometer. The pyrolysis station uses infrared radiation to heat a microgram sample to several hundred degrees C. The transfer line is a heated fused silica capillary approximately three meters in length. The membrane present in the valving system operates similarly to the early GC/MS membranes to remove the carrier gas and transmit the organics into the vacuum manifold. A low capacity ion-getter pump was used on the system without a roughing pump. The system is highly integrated, completely field portable, and operates on 24 volts dc.

A standard version of the CBMS (Army #CM7) consistent with the 1993 delivery time was tested as received for detection limits. To facilitate ease of sample introduction, a Hewlett Packard 5890 Series II gas chromatograph was interfaced to the CBMS using the standard transfer line. It was necessary to disconnect the transfer line from the pyrolyzer and attach it to a capillary column using a low dead volume Swaglock union. The column was a 0.32mm x

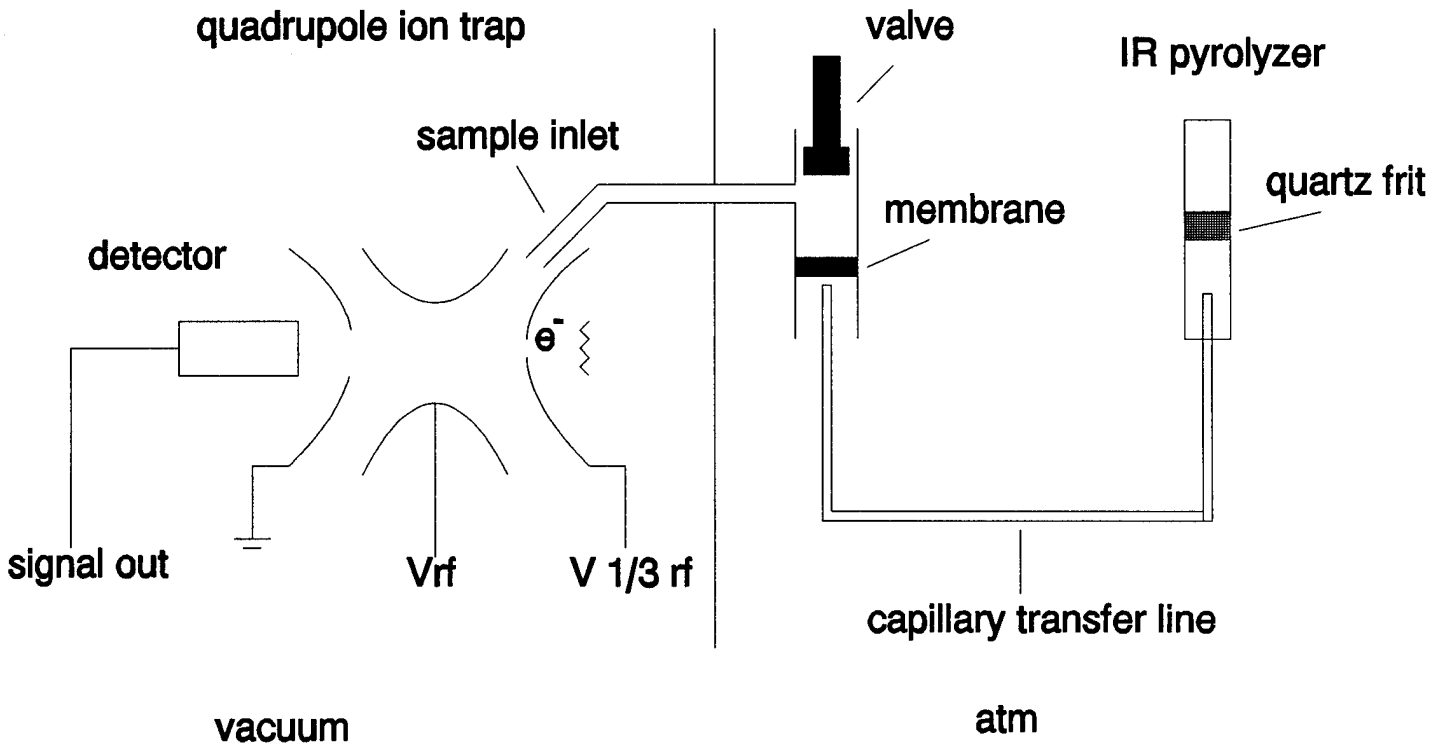

Figure 1. Chemical-Biological Mass Spectrometer (CBMS, Bruker-Franzen, Bremen, Germany) instrument diagram.

25m DB-5MS fused silica capillary column. The gas chromatograph was operated in a splitless injection mode (injector temperature 225°C) using an isothermal column temperature of 175°-225°C. A standard transfer line temperature of 200°C was used for all measurements.

Test compounds used to evaluate the sensitivity of the instrument included naphthalene, pyridine, and methyl salicylate. Pyridine was diluted for the measurements using acetonitrile while the other two compounds were prepared as methanol solutions. A series of serial dilutions were injected into the GC and the corresponding peak areas for each compound determined by using total ion chromatogram peak areas as well as selected ion peak areas. The detection limits were obtained by extrapolating the linear calibration curve to a signal-to-noise (S/N) ratio of 2.

The plotted data for the dilution experiments are shown in Figure 2. The extrapolated detection limits for the three test compounds are summarized in Table I. The detection limits

Table I. Detection Limits of Three Test Compounds on the Unmodifed CBMS.

Compound	Detection Limit
Naphthalene	0.2 pmol (27 pg)
Pyridine	25.0 pmol (2.0 ng)
Methyl Salicylate	31.0 pmol (4.7 ng)

and differential sensitivity for polar and non-polar compared favorably to those later reported by Bruker Franzen. Although considerable effort has been made by CSM, Oak Ridge National Laboratories, and Bruker Franzen, the polar/non-polar differential sensitivity effect is not understood.

Helium and nitrogen were separately used as carrier gases in the sensitivity studies. The helium carrier gas created pumping problems for the ion-getter pump and was discontinued after a few initial experiments. The data listed in Table I were therefore all from experiments using

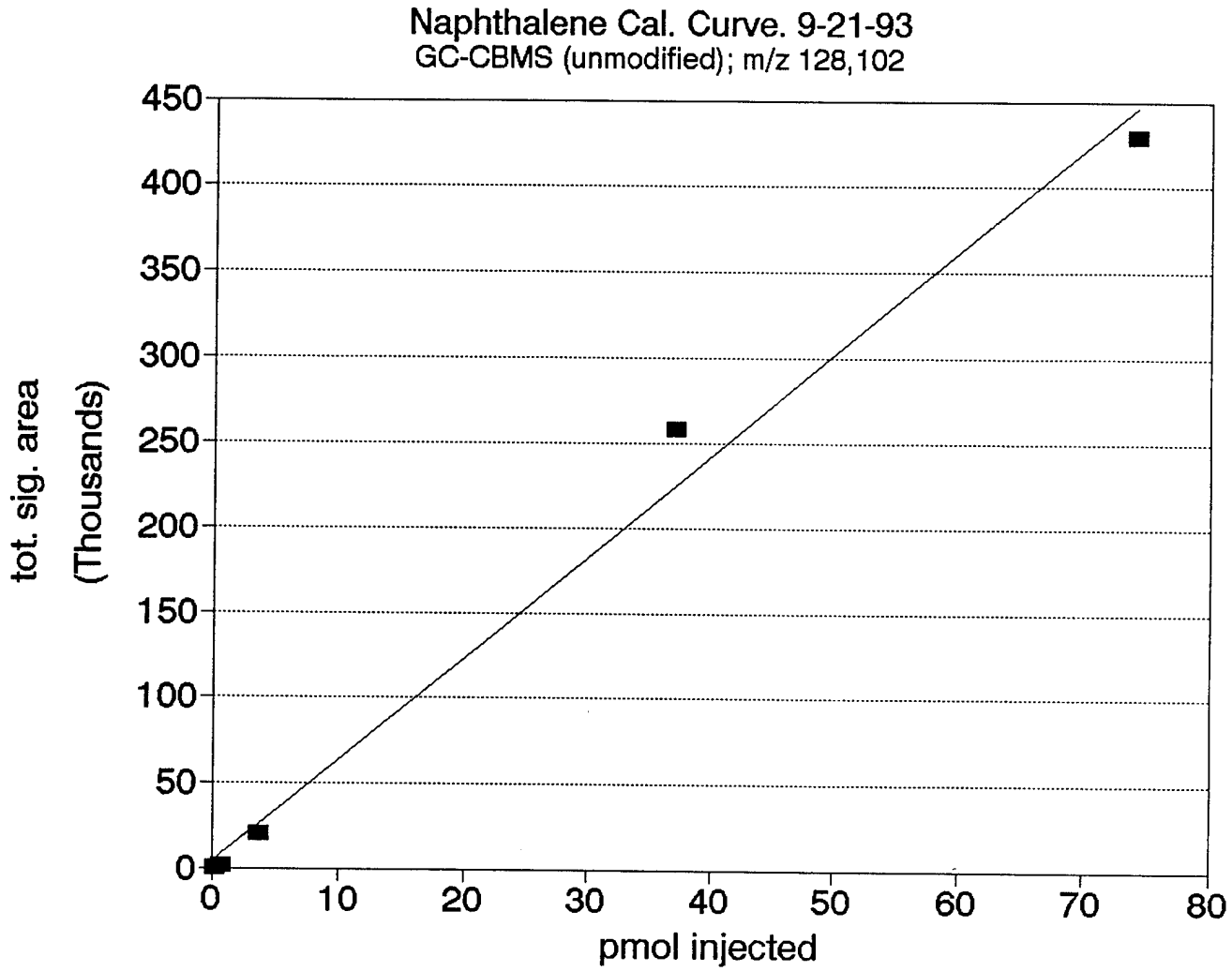

Figure 2. Extrapolated Detection Limit Data for Naphthalene.

nitrogen as the carrier gas. Additional experiments were conducted using air as the buffer gas by injecting directly into the pyrolyzer. Although the sensitivity decreased, the same polar effect was observed with air as the carrier and buffer gas. The values listed in Table I are optimum values. If the GC was used without any separation between the solvent and analyte, poorer sensitivity was observed. The best sensitivity was observed when separation occurred between the solvent and the analyte.

2. Modified CBMS

The major modification to the CBMS was the transfer of the ion trap to a new vacuum manifold that would allow evaluation of the system without pumping restraints. Figure 3 shows the mechanical drawings for the new manifold. The manifold was constructed from 3/4 " stainless steel. All joints were heliarced. The glass top and the flanges were sealed with silicon rubber o-rings. The view glass was vapor coated with iron oxide and gold to make a conducting glass surface. A 145 L/sec Leybold Turbovac 151 turbo-molecular pump was connected to the manifold. This pump has sufficient pumping capacity to allow chemical ionization and direct introduction of the transfer gas. The pumping speed was controlled using a restrictor at the mouth of the pump. An ion gauge was added to monitor manifold pressure.

Reports from Bruker Franzen stated that the short quartz transfer line from the membrane to the trap was not heated well enough to allow low volatile compounds into the MS. A brass heater encompassing the transfer line was added during the modification of CM7 that allowed transfer line temperatures up to 200°C. This heater was later removed by Bruker personnel attempting to correct a grounding problem.

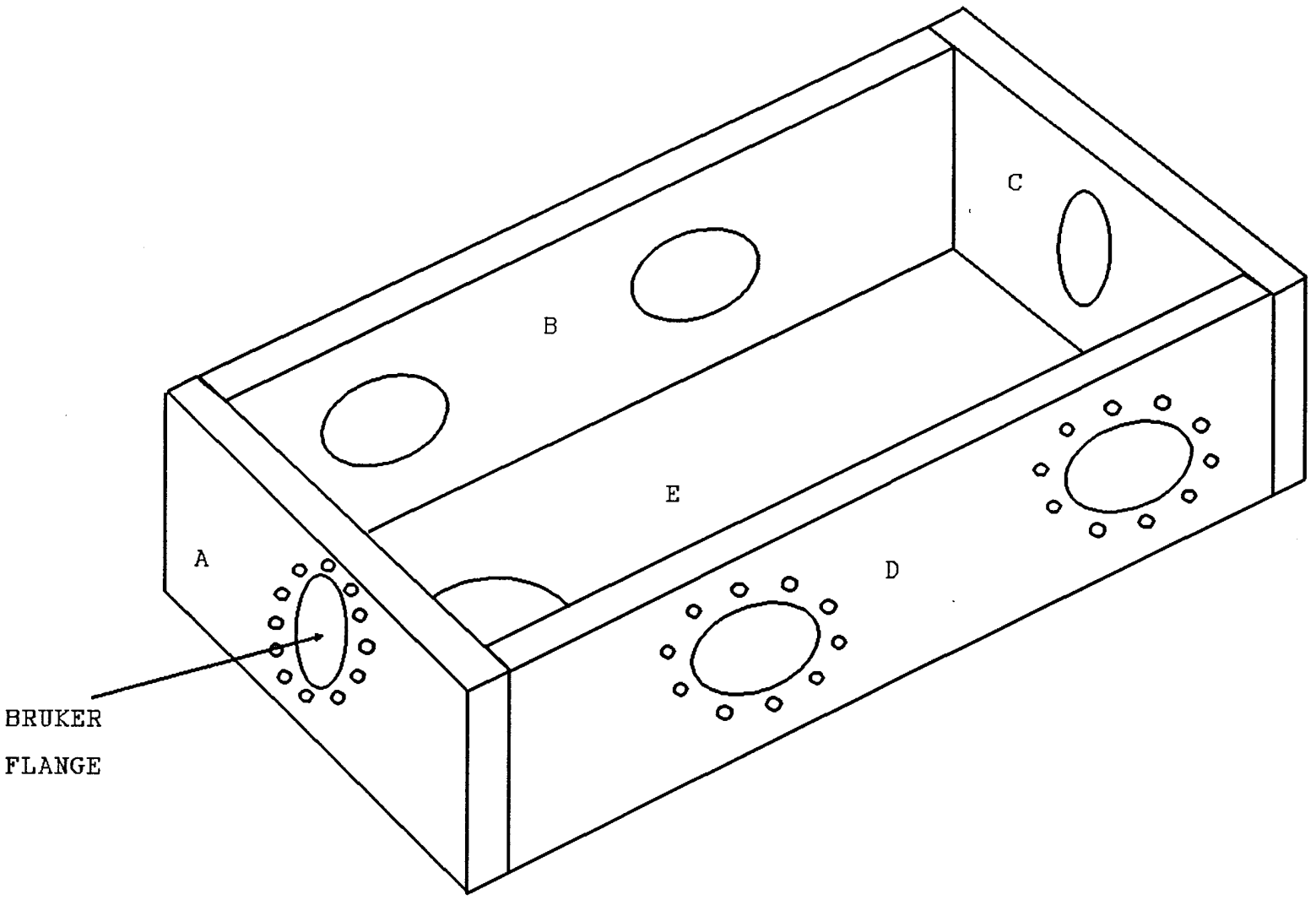

Figure 3. Vacuum Manifold for CBMS, 3-D View.

Table II. Detection Limit Results for the Modified System.

Compounds	Detection Limit
Pyridine	63 pmol (4.9 ng)
Naphthalene	0.9 pmol (120 pg)
Methyl Salicylate	6.0 pmol (900 pg)

in general, were about the same as the unmodified system.

B. Task 2 Pyrolysis with the Trap.

By the time that the modification and initial tests had been completed, the Army had selected egg albumin for their inter-laboratory standard for comparing the various CBMS instruments. A standard pyrolysis-mass spectrum of egg albumin collected on CM2 by ERDEC is illustrated in Figure 4. The overall appearance of this spectrum does not resemble typical pyrolysis-mass spectra of biomaterials from other types of pyrolysis-mass spectrometers. For example, Figure 5 represents the Py-mass spectrum of egg albumin obtained on an Extrel Curie point pyrolysis-triple quadrupole MS [1]. It is clear that certain peaks such as the m/z 77 are amplified in the CBMS, while other peaks, such as the m/z 84 peak are suppressed. Our assessment of the differences between the two spectra is that the membrane selective transmission and the differential sensitivity amplify some pyrolysis products while hindering others. It should be noted that the time scale for ionization and analysis is different for these two mass spectrometers. The time involved in the ion trap is at least 50 ms, which allows ample time for ion-molecule reactions and collision induced dissociation (CID) to occur [2,3].

The pyrolysis-mass spectrum obtained on the modified CM7 instrument of egg albumin is shown in Figure 6. The most apparent feature that distinguishes the standard spectrum (CM2, Figure 4) from this Figure is the added high masses in Figure 6. In addition, the m/z 77, 103,

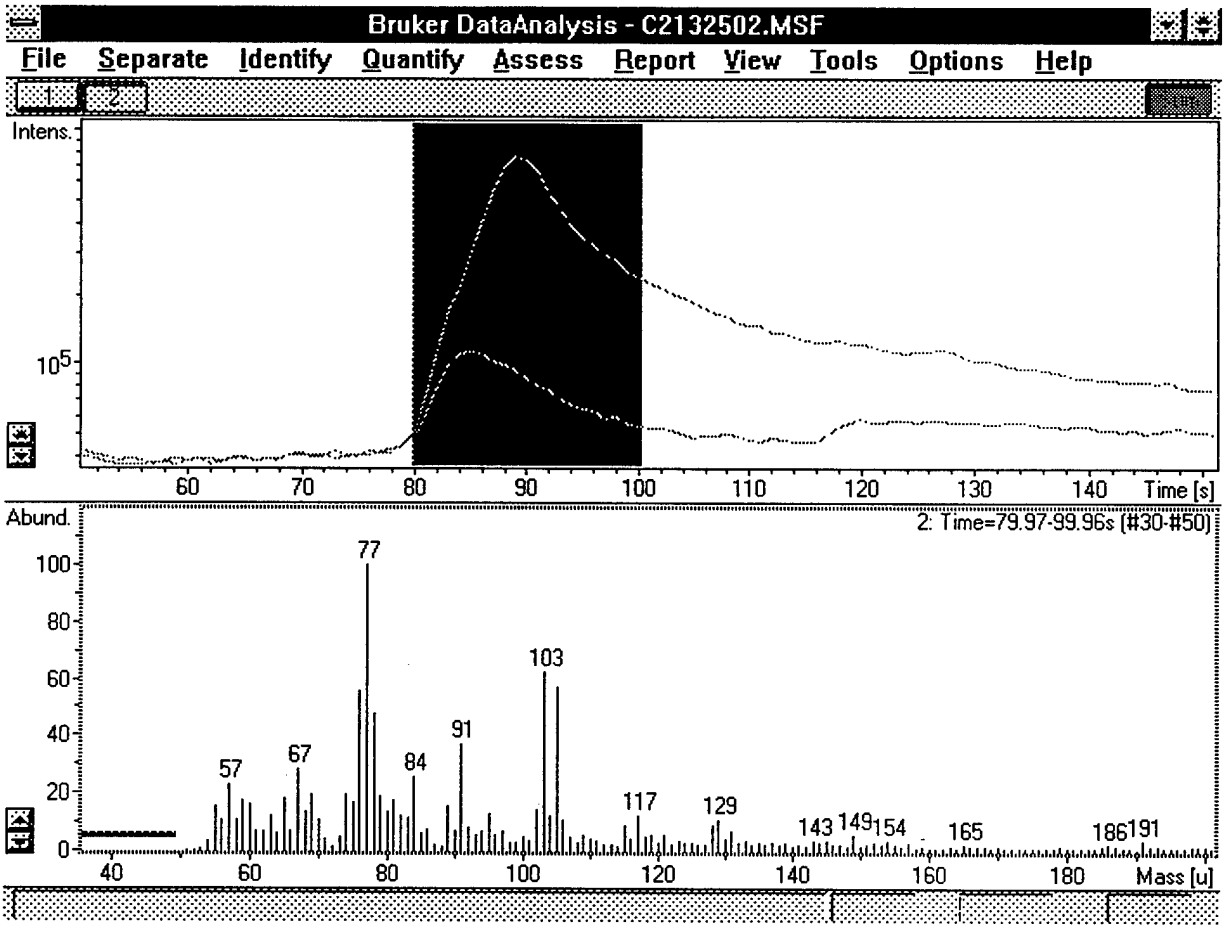

Figure 4. A Standard Pyrolysis-Mass Spectrum of Egg Albumin Measured on CM2.

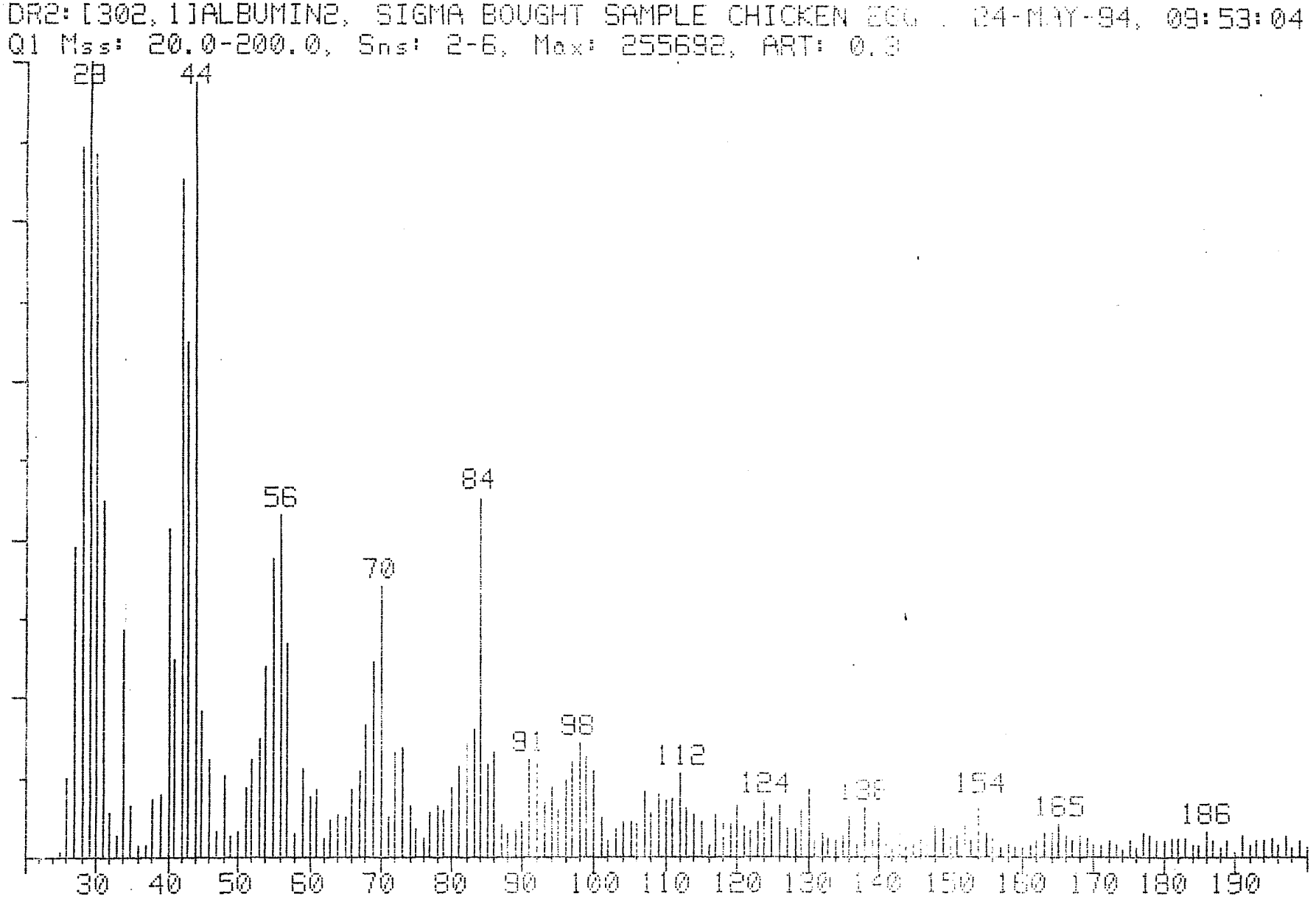

Figure 5. Pyrolysis-Mass Spectrum of Egg Albumin Measure on the Extrel Triple Quadrupole Instrument.

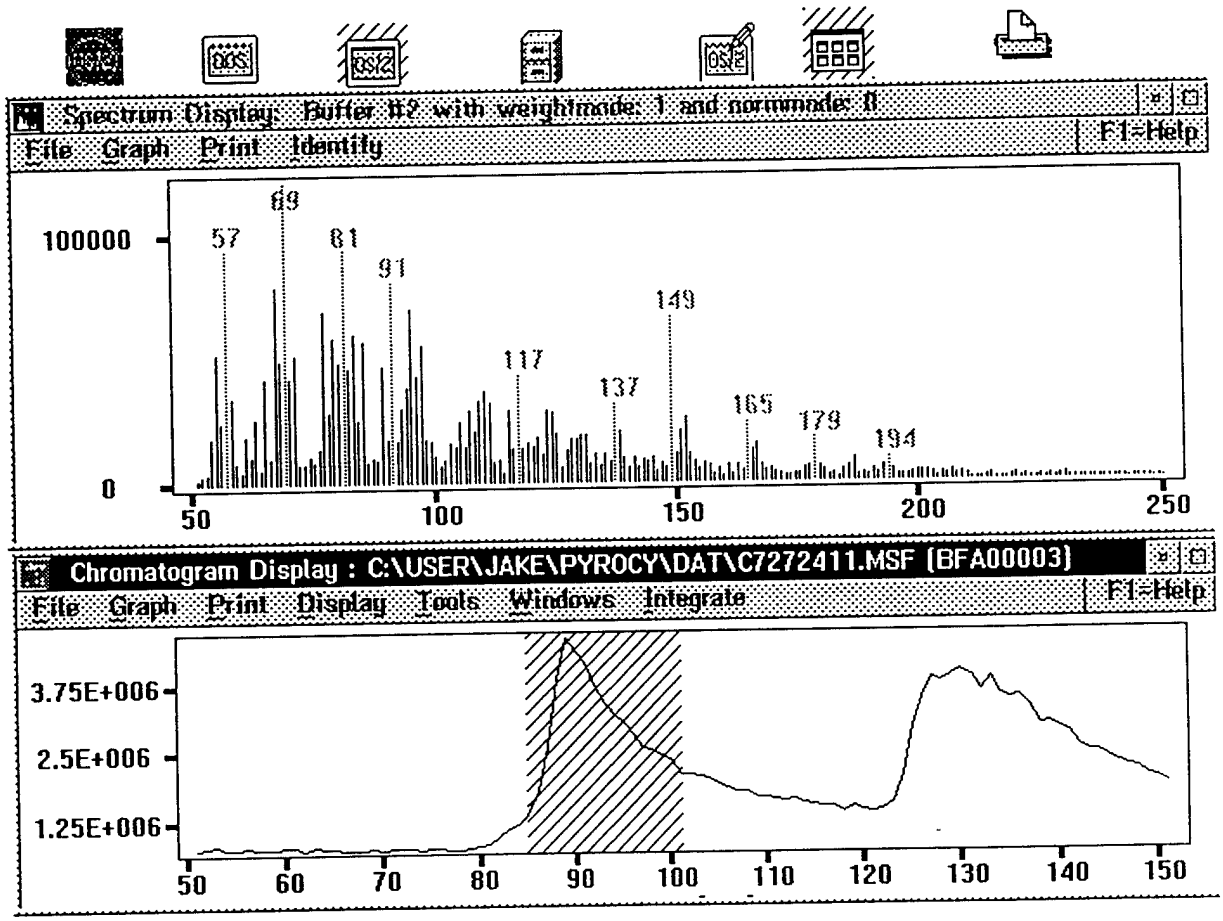

Figure 6. Egg Albumin Pyrolysis-Mass Spectrum Obtained on the CM7 Instrument.

and 105 peaks are greatly reduced in intensity in the spectrum from the CM2 instrument.

Sensitivity test were done on the modified system using the full pumping capacity of the pump. Table II summarizes the results for the three test compounds using the same solvent system as previously described. The detection limits for the modified system, peaks at m/z 110, 123, 137, 151, 165, and 179 have been determined by pyrolysis studies on the triple quadrupole mass spectrometer to be related and can be produced for a parent ion scan of m/z 95 from most bacteria. High resolution mass spectrometry is presently being done to establish the identity of this series of ions.

Extensive experiments to determine the absence of spectral agreement between the CM7 instrument and other CBMS instruments have been conducted. The temperature profile of the pyrolysis event was carefully measured on CM7 using a micro-thermocouple. After comparing the CM7 heating profile to another CBMS (CM2), adjustments were made to CM7 such that both instruments had a common heating profile. This adjustment to CM7 caused lower total ion counts, but did not change the appearance of the egg albumin spectrum.

The baseline on the modified instrument had a negatively sloped baseline. It was felt this might be influencing the high mass end of the spectrum through the charge control mechanism. This was corrected by improving the grounding on the detector and shortening the RF feed-throughs. After this correction, the baseline was found to fluctuate, which could also affect the charge control mechanism. CSM personnel determined that a faulty ground in the CBMS transfer-line power supply was responsible and corrected this by added an external DC power supply. These changes affected the ratio of high mass to low mass peaks, but did not significantly affect the masses observed.

Some of the background peaks were speculated as arising from decomposition of a materials contained in the pyrolyzer, transfer line, or valve. To improve the background, a continual nitrogen purge was added to the entire system. The high mass egg albumin peaks decreased after the nitrogen was continuously present for about 2 weeks.

The pressure inside the ion trap was varied between $2x10^{-6}$ and $6x10^{-6}$ torr using nitrogen as the buffer gas. The increased pressure did not change the peak ratios, but merely increased the relative intensity of all peaks in the spectra. This observation has been attributed to an increase in trapping efficiency with increasing buffer gas pressure [4]. Figure 7. shows two spectra taken at different pressures. The expected pressure effect on transmission of different pyrolysis products through the membrane was not observed, therefore, pressure only affected factors as the ion cloud cooling time (scan delay) and ionization time required in the trap. This behavior emphasizes the fact that the pressure pulse from the pyrolysis of the sample, the quantity of sample, and instrument parameters all must be balanced and controlled for optimum mass spectral performance.

Analyses using constant ionization time and the charge control have been conducted. Both modes produced similar spectra to the spectrum in Figure 6. This indicates that space-charge limitations were not affecting the data collected in the charge control mode. Example spectra are illustrated in Figure 8.

The effect of the amount of egg albumin pyrolyzed on the appearance of the pyrolysis-mass spectra has been investigated. Concentration levels of 5, 15, 30, and 50 mg/mL were prepared and pyrolyzed on several different days. A constant volume of 1 μL was injected into the pyrolyzer. The same parameters for the pyrolyzer and mass spectrometer were used for all

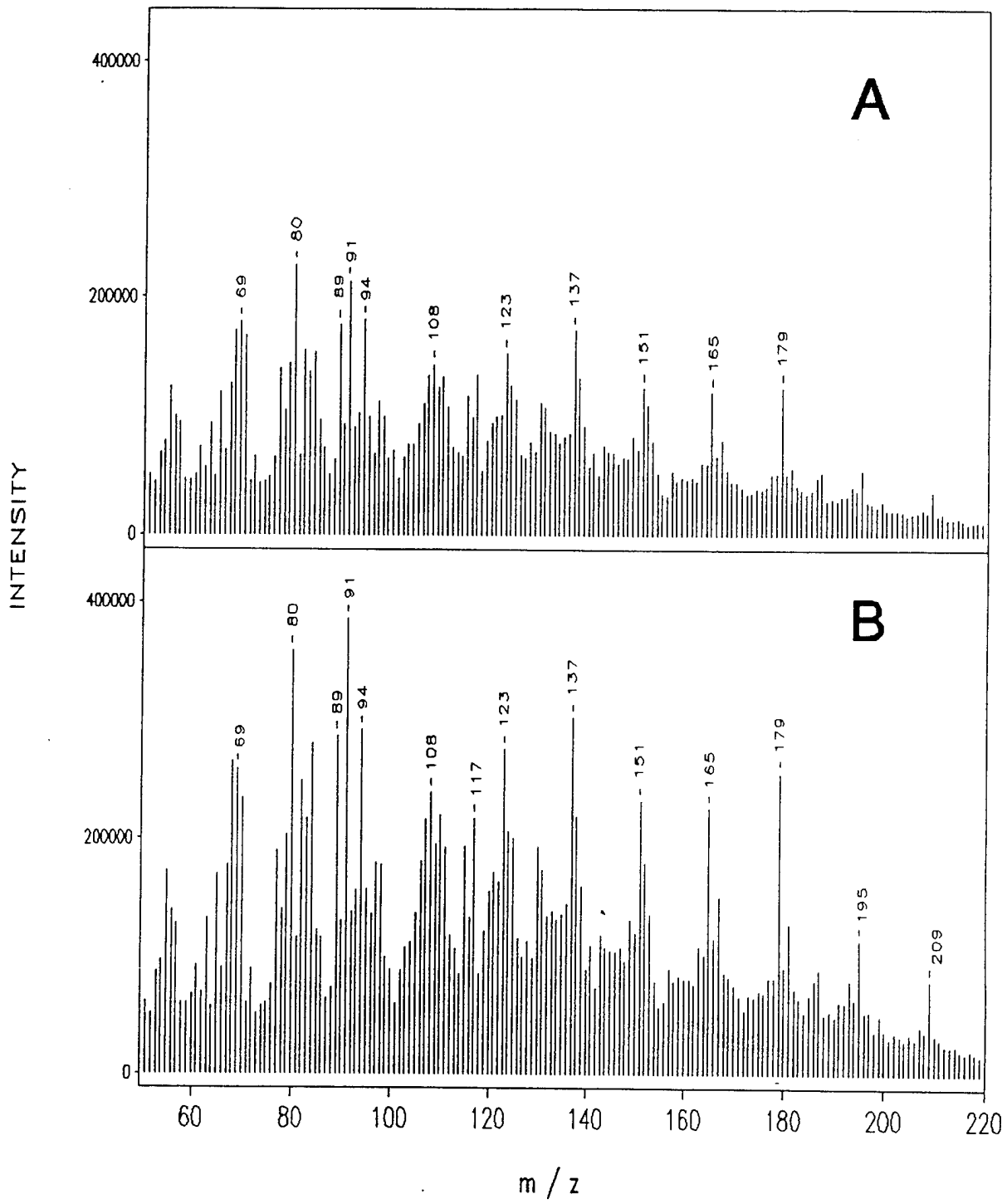

Figure 7. Egg Albumin (1 μL injected of 52 mg/mL solution), 4 injection average. Manifold pressure of A. 2×10^{-6} torr and B. 6×10^{-6} torr. Spectra: egav701a, egav701b.

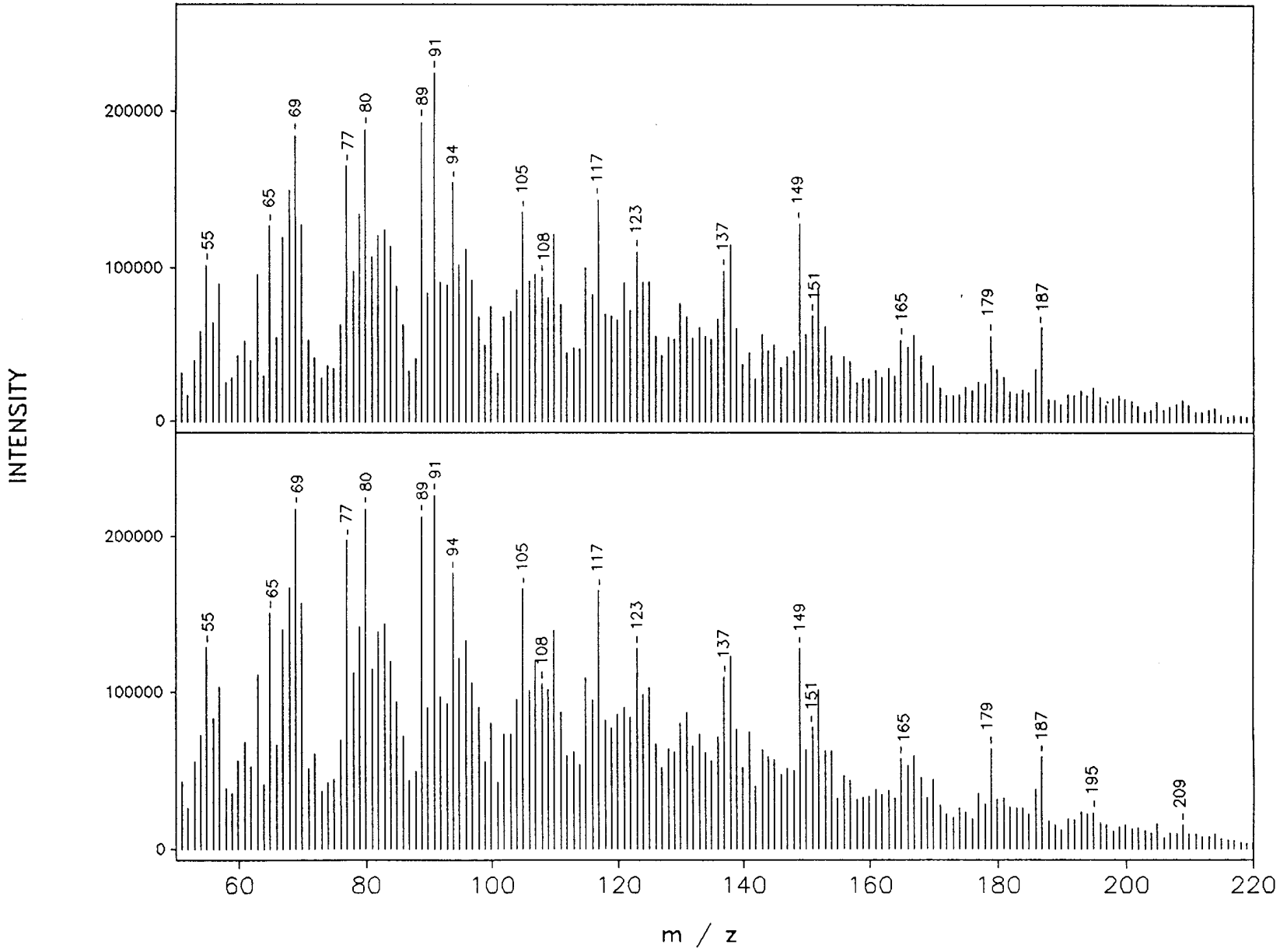

Figure 8. Comparison of egg albumin spectra using charge control (top) and constant ionization time (bottom).

analyses. All of these spectra were quite similar despite the fact that several components of the system had been replaced during the study, i.e., pyrolyzer, membrane, transfer line, and gascon pump.

Following conversion of the mass spectral data files into ASCII, factor analysis was performed on the data using the RESOLVE software package. The factor-factor plot of the data is shown in Figure 9. Along component 1 (factor 1), the no injection samples (n), the blank water injections (b), and the 5 mg/mL injections plot have about the same scores. All three samples of egg albumin above 5 mg/mL (B, C, and D) produced spectra that were very similar. The 5 mg/mL sample spectra were most similar to the CM2 spectra measured at Dugway Proving Grounds using a 50 mg/mL solution. The higher concentration spectra differed form the blanks and the 5 mg/mL sample mainly in the high mass region of the spectra. This was confirmed in the loading spectra generated as well as in the spectra themselves shown in Figure 10.

Because a slight decrease in sensitivity was observed in the modified system, the effect of pumping speed on the egg albumin spectral quality has been investigated. Pumping restrictors were designed to cover the throat of the pump. Three restrictors with speeds of approximately 50 L/s, 8.5 L/s and 4 L/s were tested. No effect was observed with the 50 L/s restrictor. The spectra collected with the 8.5 L/s restrictor in place were the most similar to the CM2 spectra of any produced on the CM7 instrument. A representative spectrum of egg albumin at 8.5 L/s is shown in Figure 10. In comparing this spectrum to the CM2 spectrum (Figure 4), there remains several important differences. These include a peak observed at m/z 69, and the lack of peaks at m/z 65 and 129.

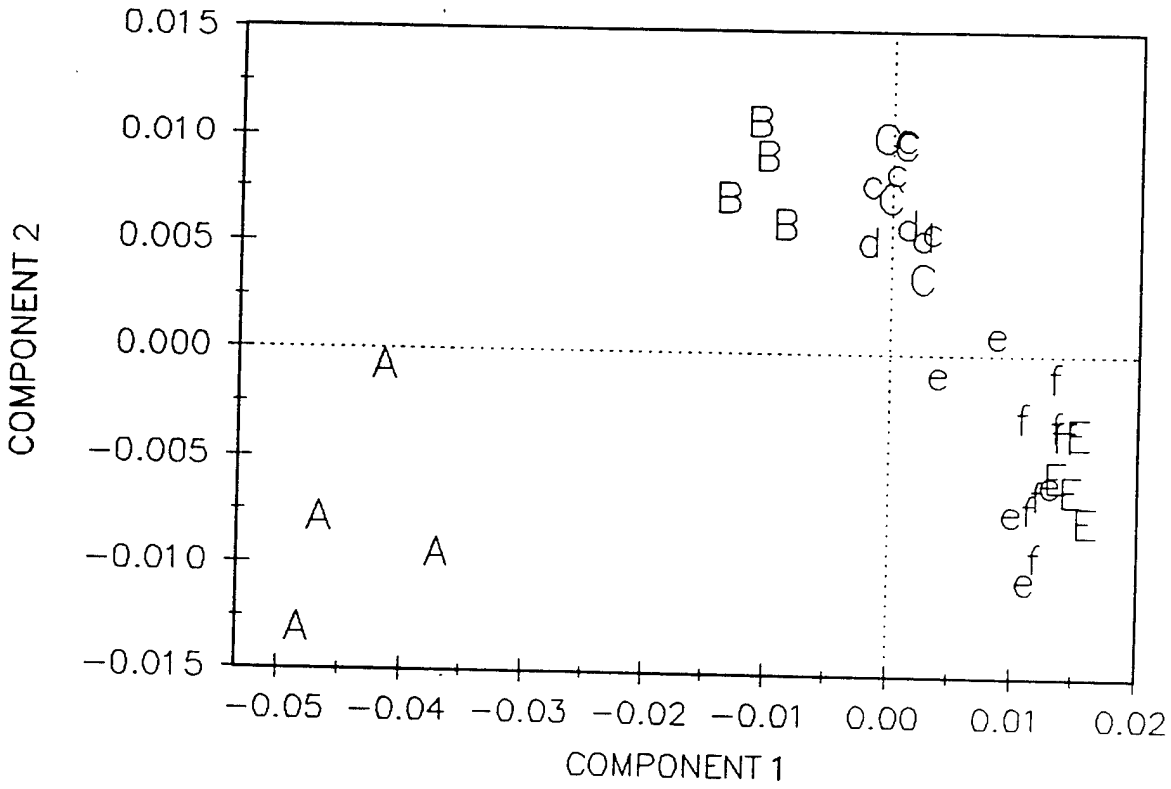

Figure 9. Principal component analysis of CBMS Egg Albumin spectra. Also included are varying manifold pressure conditions (small letters). A = 5.6 mg/mL, B = 16.1 mg/mL, {c,C,d} = 30.7 mg/mL, {e,E,f} = 52 mg/mL.

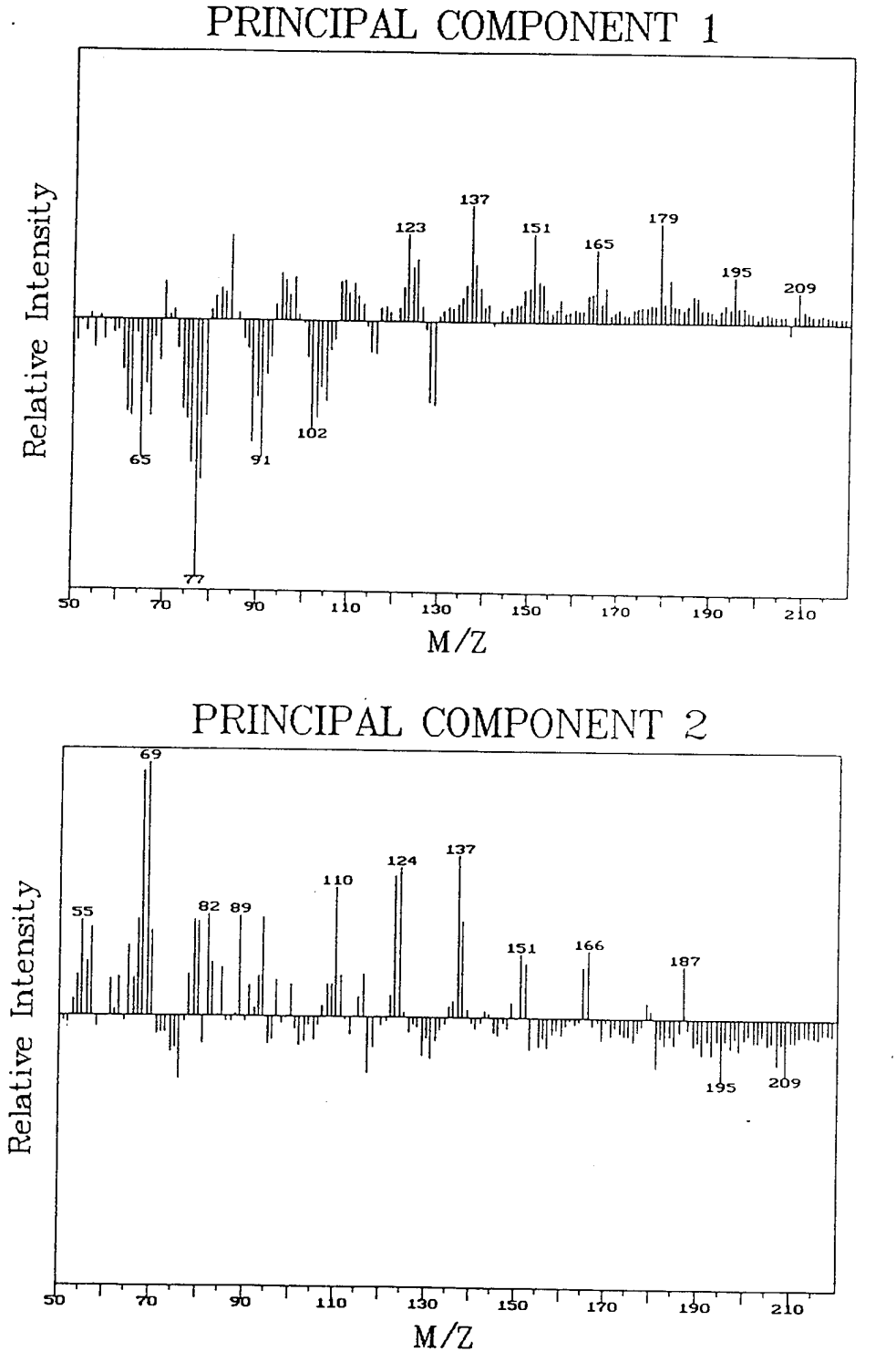

Figure 10. Loading plots for principal component analysis shown in Figure 3.

The Bruker Franzen field engineers also executed several modifications to the system. These included changing the grounding configuration and flow characteristics through the transfer line/pump as well as replacing the transfer line and pyrolysis station. A "blowback" peak caused by pulling air form the purge line that had not been previously observed, occurred after the pyrolysis station change. Overall, after the Bruker team modifications, the total ion current for a given amount of material increased; however, the major peaks that were present in data collected on CM7 were unchanged. This included the high mass peaks.

A peak at m/z 149 consistent with phthalate plasticizers had been observed in most CM7 spectra. This peak varied unpredictably in relative intensity as different conditions were investigated although it was most prevalent at low pumping speeds. Several unsuccessful modifications were made in order to eliminate this apparent contamination. One of these was to replace the specially coated glass cover with an un-coated glass. This did eliminate the m/z 149 peak over time.

C. Task 3. Long Term Reproducibility

The work for Task 3 was not started during the funding period because of the observed differences between CM7 and the other CBMS instruments. A unmodified CBMS was received at the end of the contract for the purpose of determining the long term reproducibility of the that instrument.

III. CONCLUSIONS

A CBMS was modified to include a new vacuum manifold and turbomolecular pump. Sensitivity measurements on the modified instrument clearly showed that nonpolar molecules

were detected at much lower levels than polar molecules. Although several experiments were conducted to define the factors affecting the differential sensitivity, no answer was found.

A comparison of the spectra generated on CM7 for egg albumin to the spectra from other CBMS instruments showed a different distribution of peak intensities. None of the changes in either the instrumental or pyrolysis parameters has allowed for a match between the egg albumin spectra from the modified instrument to the other versions of the CBMS. The egg albumin spectra generated on the modified instrument at low pumping speeds came closest to the standard egg albumin spectrum. The modified instrument is the only CBMS to generate egg albumin spectra which resembled pyrolysis mass spectra generated on a Curie-point pyrolysis-mass spectrometer.

The results from this investigation show that there are many operating parameters on the CBMS that need further investigation. Many of these ideas have been transmitted to Bruker Franzen.

IV. REFERENCES

1. S.J. DeLuca; E.W. Sarver; P. de B. Harrington; K.J. Voorhees; Anal. Chem., **62**, 1465 (1990).

2. N. Mechin; J. Plomley; R.E. March; T. Blasco and J.-C. Tabet; Rapid Commun. in Mass Spect., **9**, 5 (1995).

3. D.J. Harvey; OMS, **28**, 287 (1993).

4. J.S. Brodbelt; "Effects of Collision Cooling on Ion Detection," in Practical Aspects of Ion Trap Mass Spectrometry, edited by R.E. March and J.F.J. Todd, CRC Series, Vol. 1, Chapter 5., 1995, p 211.